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(54) Title: GENOMIC AMPLIFICATION WITH DIRECT SEQUENCING

(57) Abstract

Polymerase chain reaction (PCR) based sequencing method called genomic amplification with direct sequencing (GAWTS) which bypasses subcloning and increases the rate of sequence acquisition by at least fivefold. The method involves the attachment of a phage promoter onto at least one of the PCR primers. The segments amplified by PCR are transcribed to further increase the signal and to provide an abundance of single-stranded template for reverse transcriptase mediated dideoxy sequencing. An end-labeled reverse transcriptase primer complementary to the desired sequence generates the additional specificity required to generate unambiguous sequence data.

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GENOMIC AMPLIFICATION WITH DIRECT SEQUENCING

BACKGROUND OF THE INVENTION

Recently, Scharf et al., Science, 233, 1076 (1986) reported a rapid method of sequencing an allele in a region of known sequence. The method involved amplification with polymerase chain reaction (PCR) and subcloning into M13 phage. However, a need exists for a method for sequencing genomic fragments which bypasses subcloning and exhibits increased sequence acquisition.

SUMMARY OF THE INVENTION

I have invented a PCR-based sequencing method called genomic amplification with transcript sequencing (GAWTS) which bypasses cloning and increases the rate of sequence acquisition by at least fivefold. The method involves the attachment of a phage promoter onto at least one of the PCR primers. The segments amplified by PCR are transcribed to further increase the signal and to provide an abundance of single-stranded template for reverse transcriptase mediated dideoxy sequencing. An end-labeled reverse transcriptase primer complementary to the desired sequence generates the additional specificity required to generate unambiguous sequence data.

GAWTS can be performed on as little as a nanogram of genomic DNA. The rate of GAWTS can be increased by coamplification and cotranscription of multiple regions as illustrated by two regions of the factor IX gene.

Since GAWTS lends itself well to automation, further increases in the rate of sequence acquisition can be expected. Further, commercial applications of GAWTS include: (1) the generation of a kit to assist

others utilize the technique; (2) the generation of an instrument that automates the method; and (3) the generation of diagnostic tests that utilize the method.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a diagramatic representation of the amplification and direct sequencing method of the present invention.

Figure 2 shows a region of factor IX gene and indicates the location of the PCR primers and the reverse transcriptase primer to sequence one region of factor IX gene of the present invention.

Figure 3A shows an agarose gel after 27 cycles of polymerase-chain reaction.

Figure 3B shows the subsequent transcription reaction in accordance with the present invention.

Figure 3C shows an autoradiogram of a segment of sequencing gel in accordance with the present invention.

Figure 4 shows the results of genomic amplification with direct sequencing with simultaneous amplification and transcription of a 331bp region in the amino acid coding segment of exon 8, and a 250 bp region which begins 1.2 kb downstream in exon 8.

DETAILED DESCRIPTION OF THE INVENTION

In contrast to autosomal recessive mutations, deleterious X-linked mutations are eliminated within a few generations because the affected males reproduce sparingly if at all. Thus, each family in an X-linked disease such as hemophilia B represents an independent mutation. From the perspective of efforts to understand the expression, processing, and function of factor IX, this is useful since a large number of muta-

tions are potentially available for analysis. In addition to facilitating structure-function correlations, the rapidity of GAWTS makes it practical to perform direct carrier testing and prenatal diagnosis of at-risk individuals. By amplifying and sequencing 11 regions of the hemophilic factor IX gene which total 2.8 kb, it should be possible to delineate the causative mutation in the overwhelming majority of individuals as these regions contain the putative promoter, the 5' untranslated region, the amino acid coding sequences, the terminal portion of the 3' untranslated region, and the intron-exon boundaries. Once the mutation is delineated, GAWTS can be used to directly test an at-risk individual, thereby finessing the multiple problems associated with indirect linkage analysis.

GAWTS depends on two types of sequence amplification and a total of three oligonucleotides to generate the needed specificity (see Fig. 1). The steps of GAWTS as shown in Fig. 1 are as follows:

A. The region of genomic DNA to be amplified is indicated by the open rectangle. Two strands with their 5' to 3' orientation are shown. The darkened regions represent flanking sequences.

B. The oligonucleotides anneal to sites just outside the sequence to be amplified. One of the oligonucleotides has a 29 base T7 promoter sequence.

C. PCR consists of repetitive cycles of denaturation, annealing with primers, and DNA polymerization. Since the number of fragments with defined ends increases much faster than the number with undefined ends, virtually all the fragments are of defined size after 27 cycles. However, since the oligonucleotides anneal to other sites in the genome, multiple spurious fragments are also amplified. The

segment pictured is a specifically amplified sequence.

D. RNA is transcribed from the T7 promoter. This provides a convenient source of single-stranded nucleic acid for dideoxy sequencing.

E. Due to the complexity of the mammalian genome, the amplified and transcribed sequences contain other genomic segments whose flanking sequences cross-hybridize with the PCR primers at the stringency generated by the DNA polymerization reaction. As a result, another level of specificity is crucial to obtaining interpretable sequences. That specificity is provided by utilizing an oligonucleotide primer for reverse transcriptase which lies in the region of interest.

F. Reverse transcriptase is used to generate sequence data by the dideoxy method.

The first region chosen for amplification was part of the amino acid coding region of exon 8 of the factor IX gene. Figure 2 shows the relevant sequence and indicates the locations of the PCR primers and the reverse transcriptase primer. Primers are named using the numbering scheme in Yoshitake et al., Biochemistry 24, 3736 (1985).

A. Oligonucleotides synthesized (Synthetic Genetics, Inc.) for GAWDS of a region in the proximal part of exon 8.

The PCR primers are (T7-29)-E8(30884)-48D and (PST1-9)E8(31048)-27U and the reverse transcriptase primer is E8(31025)-17U. The noncomplementary bases in E8(31048)-27U may be ignored as they are not relevant to this series of experiments. Note that by replacing these bases with a different phage promoter, it should

be possible to generate an amplified fragment where both strands could be selectively transcribed and sequenced.

Since oligonucleotides tend to rapidly accumulate when using GAWTS, it is helpful to have informative names. The notation used above is one of the form: (identifier for noncomplemntary 5' base-length) region of the gene (location of the 5' complementary base using the numbering system of Yoshitake et al., supra) - total size and 5' to 3' direction of the oligonucleotide. The region of the gene can be abbreviated by Upstream, Exon number, Intron number, and Downstream. The direction of the oligonucleotide is either Upstream or Downstream relative to the direction of the transcription. Thus, (T7-29)E8(30884)-48D has a T7 promoter (plus a 6 base clamping sequence) of 29 bases. It is complimentary to a sequence that in exon 8 begins at base 30884. The oligonucleotide is a 48 mer which heads downstream relative to E9 mRNA. E8(310 25)-17U is also located in exon 8, lacks a 5' non-complementary sequence and begins at 31025. It is a 17 mer that heads upstream. Likewise, U (-140)-16U is a 16 mer located upstream of the gene which begins at base -140 and heads further upstream of the gene.

B. GAWTS for exon 8 of the factor IX gene utilizing the primers pictured in Fig. 2.

1. METHOD: The PCR, transcription, and sequencing reaction were performed as previously described with minor modifications. See R.K. Saiki, et al., Nature 324, 163 (1986); D.A. Melton, et al., Nucleic Acids Res. 12, 7035 (1984); J. Geliebter, Focus 9:(1)5-8(1987). In brief, a microfuge tube containing

1 ug (10 ng/ul) of DNA was denatured at 95°C for 10 min (2 min in subsequent cycles) in the presence of the following: 50 mM sodium chloride, 10 mM Tris-HCl pH 7.6, 10 mM magnesium chloride, 10% DMSO, and 1.5 mM of each of the four deoxynucleotide triphosphates. After microfuging, samples were then annealed at 50°C for 2 min and subsequently one-half unit of Klenow fragment was added. Samples were incubated at 50°C for another 2 min. Twenty-six additional cycles of denaturation, annealing, and polymerization were performed.

It is crucial to assure that the Klenow fragment added at later cycles has the same activity as that added at early cycles. To this end, fresh aliquots of Klenow fragments were removed from the -20°C freezer every seven cycles and diluted from the manufacturer buffer to 10 u/l with dilution buffer (10 mM Tris pH 7.5, 1 mM DTT, 0.1 mM EDTA, and 1.5 mM of the four deoxytriphosphates).

After a final denaturation, 3 ul of the amplified material was added to 17 ul of the RNA transcription mixture: 40 mM Tris-HCl pH 7.5, 6 mM magnesium chloride, 2 mM spermidine, 10 mM sodium chloride, 0.5 mM of the four ribonucleotide triphosphates, 1.6 U/ul RNAsin, 10 mM DTT, 10 U T7 RNA polymerase, and DEPC treated H_2O. Samples were incubated for 1 hr at 37°C and the reaction was stopped with 5 mM EDTA.

For sequencing, 2 ul of the transcription reaction and 1 ul of the 32p end labeled (see below) reverse transcriptase primer were added to 10 ul of annealing buffer (250 mM KCl, 10 mM Tris-HCl pH 8.3). The samples were heated at 80°C for 3 min and then annealed for 45 min at 45°C (approxmately 5°C below the denaturation temperature of the oligonucleotide).

Microfuge tubes were labeled with A, C, G, and T. The following was added: 3.3 ul reverse transcriptase buffer (24 mM Tris-HCl pH 8.3, 16 mM magnesium chloride, 8 mM DTT, 0.4 mM dATP, 0.4 mM dCTP, 0.8 mM dGTP, and 0.4 mM dTTP) containing 5 U of AMV reverse transcriptase, 1 ul of either 1 mM ddATP, or 1 mM ddCTP, or 1 mM ddGTP, or 2 mM ddTTP and finally, 2 ul of the primer RNA template solution. The sample was incubated at 50°C for 45 min and the reaction was stopped by adding 2.5 ul of 100% formamide with 0.3% bromophenol blue and xylene cyanol FF. Samples were boiled for 3 min and 3 ul were loaded onto a 100 cM sequencing gel and electrophoresed for about 15,000 V-h. Subsequently, autoradiography was performed, utilizying known techniques.

End-labeling of the reverse transcriptase primer was performed by incubating a 0.1 ug sample of oligonucleotide in a 13 ul volume containing 50 mM Tris-HCl (ph 7.4), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 100 uCi [-32p] ATP (5,000 Ci/mmole) and seven units of polynucleotide kinase for 30 min at 37°C. The reaction is heated to 65°C for 5 min and 7 ul of water was added for a final concentration of 5 ng/ul of oligonucleotide. One ul of labeled oligonucleotide was added per sequencing reaction without removal of the unincorporated mononucleotide.

GAWTS for exon 8 of factor IX gene was evaluated as shown in Figure 3 for lane 1, 40 picograms (1 picogram of sequence to be amplified) of pSP6-9A, a plasmid containing factor IX cDNA, was the input DNA. For reactions 2 and 3, 1 ug of genomic DNA from a normal and a hemophiliac individual, respectively, was the input DNA. (A) 3% NuSieve/1% Seakem agarose (FMC) gel of 30% of the PCR amplified material. (B) 3%

NuSieve/1% Seakem agarose gel of the transcribed material. The unlabeled lanes contain a HaeIII digest of 0X174 as size markers. (C) Autoradiogram of segment of the sequencing gel. From left to right, the order of the lanes are ATCG. In set 3, there appears to be an extra "A" at position 2 but review of the original autoradiograph clearly indicates that this is artifact.

2. RESULTS: Figure 3 shows an agarose gel after 27 cycles of polymerase-chain reaction (3A) and the subsequent transcription reaction (3B). In sample 1, the input DNA was 40 picograms of pSP6-9A, a 6.5 kb plasmid containing factor IX cDNA which was kindly provided by Dr. C. Shoemaker of Genetics Institute Inc. The total amount of the region to be amplified is approximately 1 picogram. As Fig. 3A (lane 1) shows, there was a discrete amplified fragment (predicted size: 209 bp) which migrated as expected relative to the size markers. From the intensity of ethidium bromide fluorescence relative to known size standards, it is estimated that a 500,000 fold amplification had occurred.

Amplified material (25 mg) was transcribed with T7 RNA polymerase, resulting in approximately 10 ug of transcript (3B). Ten percent of the transcribed material was then added to a reverse transcriptase sequencing reaction. Perfect agreement with the published sequence was obtained.

In sample 2, the input was 1 ug of genomic DNA from a normal individual and, in sample 3, the input was 1 ug of DNA from an individual with hemophilia B. Although spurious amplification masks the expected band, the specificity conferred by the reverse transcriptase primer allowed unambiguous sequence determination (Fig. 3C). No sequence alterations were

seen in the 115 bases of sequence which lie between the reverse transcriptase primer and the 48 base polymerase chain reaction primer.

This region was examined for an additional 38 males (6 with hemophilia B and 32 unaffected individuals from a variety of ethnic groups) and no sequence alterations were seen.

To test the sensitivity of GAWTS, the amount of genomic DNA was incrementally decreased. With the aid of an intensifying screen, a sequence could be discerned with 1 ng of input DNA (the amount of DNA contained in 150 diploid cells). At this level, PCR is possible in a crude cell lysate. (R.K.Saiki et al, supra)

As a test of the generality of the procedure, an attempt was made to amplify four additional regions of the factor IX gene: (1) a 332 bp sequence which includes the putative promoter region, exon 1, and the splice donor junction of intron 1; (2) a 315 bp region that includes exon 6 and the flanking splice junctions; (3) a 331 bp region in the amino acid coding region of exon 8; and (4) a 250 bp region that contains the distal 3' untranslated region of exon 8. In three of the four regions, the amplified regions had a band of expected size that was discernable above the background of nonspecific amplification and transcription on an agarose gel. Although the intensity of the signal varied, the four regions all produced unambiguous sequence data. Unlike previous methods which involved cloning of single molecules from a mixture, the error rate of GAWTS is relatively unaffected by the fidelity of polymerization because the sequence obtained is the dominant sequence in the population.

No point mutations or new polymorphisms were

found in the normal and hemophilic individuals analyzed by GAWTS for the regions mentioned above. However, the previously documented polymorphism in amino acid 148 in exon 6 was detected.

C. GAWTS with simultaneous amplification and transcription of a 331 bp region (Region I) in the amino acid coding segment of exon 8, and a 250 bp region (Region II) which begins 1.2 kb downstream in exon 8.

To determine whether more than one region could be simultaneously amplified with PCR and transcribed, the 331 bp region in the amino acid coding region of exon 8 and the 250 bp region in the distal 3' untranslated region of exon 8 were utilized. Both sequences could be obtained with the appropriate reverse transcriptase primer.

PCR and transcription reactions were performed on 1 ug of DNA with: (1) primers specific for Region I, (2) primers specific for Region II, and (3) both sets of primers. Sequencing was performed as follows: (A) template from PCR/transcription reaction (1) with reverse transcriptase primer specific for Region I, (B) template from PCR/transcription reaction (2) with reverse transcriptase primer specific for Region II, (C) template from PCR/transcription reaction (3) with reverse transcriptase primer specific for Region I, and (D) template from PCR/transcription reaction (3) with reverse transcriptase primer for Region II. As seen in Figure 4 the order of the lanes are ATGC.

Simultaneous amplification was also successful for a second pair of regions suggesting that it can

further enhance the rate of sequence acquisition while decreasing the cost of sample processing.

The oligonucleotides utilized above were synthesized by the phosphoramidite chemistry and subsequently gel purified. Purification is not always necessary because crude (T7-29)E8(30884)-48D, a 48 mer, gave an acceptable sequence despite the fact that gel staining indicated that less than 50% of the molecules were of the desired length.

GAWTS substantially reduces the time required to sequence an allele as eight samples can be amplified, transcribed and loaded onto a sequencing gel in an eight to nine hour day. Thus, in a span of less than two years, the rate of detection of changes in genomic sequence has increased by a factor of about 100. As a result, an array of experiments are now feasible in a diversity of fields. As there are no centrifugations, ethanol precipitations, or complicated procedures such as plaque lifts, GAWTS lends itself well to automation. With modifications of an automated PCR instrument, R.K. Saiki et al., Nature, 324, 163 (1986) and an automated sequencer, L.M. Smith et al., Nature, 321, 674 (1986), it should be possible to generate a fully automated system.

As the sophistication of the component instruments increases, it is conceivable that the rate of genomic information retrieval could be further increased by orders of magnitude. This has broad implications for both research and clinical medicine. As one example, most DNA-based analyses of tumors currently utilize easily detectable mutations such as gene amplifications and chromosome rearrangements. If it becomes possible to rapidly sequence the promoter region, exons, and splice junctions of multiple oncoge-

nes in neoplasms, it should be possible to obtain a much more comprehensive view of the genetic alterations that accompany malignancy. The insights gained may well aid the clinician in determining prognosis and optimizing therapy.

It is to be understood that the GAWTS method described herein can be adopted by one of skill in the art to provide kits to assist others utilize the technique. Also diagnostic tests that utilize the method are envisioned. One example of a kit incorporating the method of the present invention is designed to rapidly and specifically amplify nucleic acid and produce a transcript of the nucleic acid. Kit components include chain reaction oligonucleotide primers for hybridizing to each end of a nucleic acid sequence with at least one of said primers including a promoter sequence and components for amplifying said nucleic acid sequence.

While GAWTS as described herein is used to sequence gene fragments the present invention is more broadly directed to a rapid and sensitive method of amplification of nucleic acid sequences to provide for subsequent production of an RNA transcript. This involves hybridizing oligonucleotide primers to each end of a nucleic acid sequence with at least one of the primers including a promoter sequence; and amplifying the sequence with methods such as polymerase chain reaction. Subsequent generation of an mRNA transcript and sequencing of the transcript can then be conducted in accordance with the present invention. Accordingly, the nucleic acid to be amplified can be RNA or DNA.

The sensitivity of GAWTS allows the diagnosis of infectious agents including viruses such as the HIV virus, bacteria such as gonnococcus mycobacterium and mycoplasma, and eukaryotes agents such as fungi and

parasites. The sequence data will facilitate the study of the epidemiology of these agents.

The sensitivity of GAWTS can be increased by performing a number of cycles of PCR with one pair of primers and then performing subsequent cycles with a nested pair of primers complementary to sequences internal to the initial primer pair. Multiple rounds of nested PCR are possible. Applications to in situ detection of nucleic acid sequences are possible.

Many further extensions and enhancements can be envisioned. Two of particular interest involve the amplification of RNA such as messenger RNA. cDNA can be made by established protocols see J. Geliebter, Focus 9, 5 (1987). Then the cDNA can be amplified and sequenced as described above. Alternately, the amplified cDNA can be used for other purposes such as insertion into an expression vector or transcription followed by in vitro translation.

Previously undefined genomic sequence at the junction of a defined sequence can be obtained by a number of variations of GAWTS including the following:

1. annealing under nonstringent conditions with a specific oligonucleotide containing a promoter such as T7 (oligonucleotide A), and extending with a polymerase;

2. Elimination of the primer by a method such as ultrafilteration or gel electrophoresis;

3. Denaturation and annealing under nonstringent conditions with an oligonucleotide (oligonucleotide B) which can be enlongated with a polymerase;

4. Removal of oligonucleotide B (length of oligonucleotide B = approximately 16 nucleotides;

5. Addition of oligonucleotide B fused with

a different promoter (such as T3) (oligonucleotide C), and oligonucleotide A;

6. Transcription;

7. DNAse treatment;

8. Inactivation of DNAse;

9. Production of cDNA using oligonucleotide C and oligonucleotide D which anneals three primers to the known sequence that oligonucleotide A anneals to;

10. Amplification of cDNA with PCR

11. Transcription using the second (T3) promoter; and

12. Sequencing with reverse, transcriptase using oligonucleotide E which anneals 3 to the site of annealing of oligonucleotide D.

WHAT IS CLAIMED IS:

1. A rapid and sensitive method for generating an RNA transcript of a specific segment of genomic DNA comprising the steps of:

(a) hybridizing oligonucleotide primers to each end of a nucleic acid sequence to be amplified, with at least one of said primers including a promoter sequence; and

(b) amplifying said sequence.

2. The method of claim 1, further comprising the step (c) of generating an RNA transcript of the segment of genomic DNA.

3. The method of claim 2 further comprising the step (d) of obtaining a sequence of said RNA transcript.

4. The method of claim 1 wherein said nucleic acid to be amplified is RNA or DNA.

5. The method of claim 1 wherein said amplification involves polymerase chain reaction.

6. A genomic amplification and sequencing method comprising the steps of:

(a) hybridizing polymerase chain reaction oligonucleotide primers to each end of a gene fragment with at least one of said primers including a promoter sequence;

(b) amplifying said gene fragment with polymerase chain reaction; and

(c) sequencing said gene fragment directly by transcription of said gene fragment and subsequent

reverse transcriptase mediated dideoxy sequencing.

7. A process for genomic amplification with direct sequencing comprising the steps of:

(a) denaturing genomic DNA, including a genomic fragment to be sequenced;

(b) annealing polymerase chain reaction oligonucleotide primers to said genomic fragment with at least one of said primers including a promoter sequence;

(c) amplifying said genomic fragment with repetitive cycles of denaturing and annealing with said primers and DNA polymerization, said cycles being repeated until said amplified genomic fragments are substantially all of defined size including said genomic fragment to be sequenced;

(d) transcribing said amplified genomic fragment from said promoter to provide a single stranded template;

(e) annealing said transcribed template with an end-labeled oligonucleotide primer for reverse transcriptase, said primer being complementary to said genomic fragment to be sequenced; and

(f) conducting reverse transcriptase mediated dideoxy sequencing.

8. The process of claim 7 wherein one of said oligonucleotide primers in step (b) includes a 29 base T7 promoter sequence.

9. A process for genomic amplification with direct sequencing of regions of factor IX gene comprising the steps of:

(a) denaturing said factor IX gene;

(b) annealing polymerase chain reaction oligonucleotide primers to a region of said factor IX gene to be sequenced with at least one of said primers including a promoter sequence;

(c) amplifying said region of said factor IX gene with repetitive cycles of denaturing and annealing with said primers and DNA polymerization, said cycles being repeated until substantially all amplified fragments including said region are of defined size;

(d) transcribing said amplified region from said promoter to provide a single stranded template;

(e) annealing said transcribed template with an end-labeled oligonucleotide primer for reverse transcriptase, said primer being complementary to said region to be sequenced;

(f) conducting reverse transcriptase mediated dideoxy sequencing.

10. The process of claim 9 wherein said region to be sequenced is a part of the amino acid coding region of exon 8.

11. The process of claim 10 wherein said polymerase chain reaction oligonucleotide primers in step (b) are (T7-29)-E8(30884)-48D and (PSTI-9)E8(31048)-27U and the reverse transcriptase primer in step (e) is E8(31025)-17U.

12. The process of claim 11 wherein said region to be sequenced is shown in Fig. 2.

13. The process of claim 9 wherein said amplification and transcription are conducted simultaneously.

14. A process for genomic amplification with direct sequencing of the region of factor IX gene shown in Fig. 2 comprising the steps of:

(a) denaturing said factor IX gene;

(b) annealing polymerase chain reaction oligonucleotide primers E8(30884)-48D and E8(31048)-27U to said region of factor IX gene to be sequenced, said E8(30884)-48D including a phage promoter sequence;

(c) amplifying said region of said factor IX gene with 27 repetitive cycles of denaturing and annealing with said primers and DNA polymerase;

(d) transcribing said amplified region from said promoter to provide a single stranded template;

(e) annealing said transcribed template with end-labeled reverse transcriptase primer E8(31025)-17u;

(f) conducting reverse transcriptase mediated dideoxy sequencing.

15. The process of claim 14 wherein said phage promoter sequence is a 29 bp sequence of T7.

16. A kit to rapidly and specifically amplify nucleic acid and produce a transcript of said nucleic acid comprising:

(a) chain reaction oligonucleotide primers for hybridizing each end of a nucleic acid sequence with at least one of said primers including a promoter sequence; and

(b) components for amplifying said nucleic acid sequence.

17. The kit of claim 16 further comprising a component for generating an mRNA transcript.

18. The kit of claim 17 further comprising a component for sequencing said mRNA transcript.

19. A kit for performing genomic amplification and direct sequencing comprising:

(a) polymerase chain reaction oligonucleotide primers for attachment to each end of a gene fragment with at least one of said primers including a promoter sequence; and

(b) component for amplifying said gene fragment with polymerase chain reaction.

20. The kit of claim 19 further comprising a component for sequencing said gene fragment directly by transcription of said gene fragment and subsequent reverse transcriptase mediated dideoxy sequencing.

21. A rapid and sensitive method for obtaining unknown nucleic acid sequence without necesitating cloning said method comprising of using olgonucleotide with and without attached rna promoters and annealed under stringent and sometimes nonstringent conditions.

FIG. 1

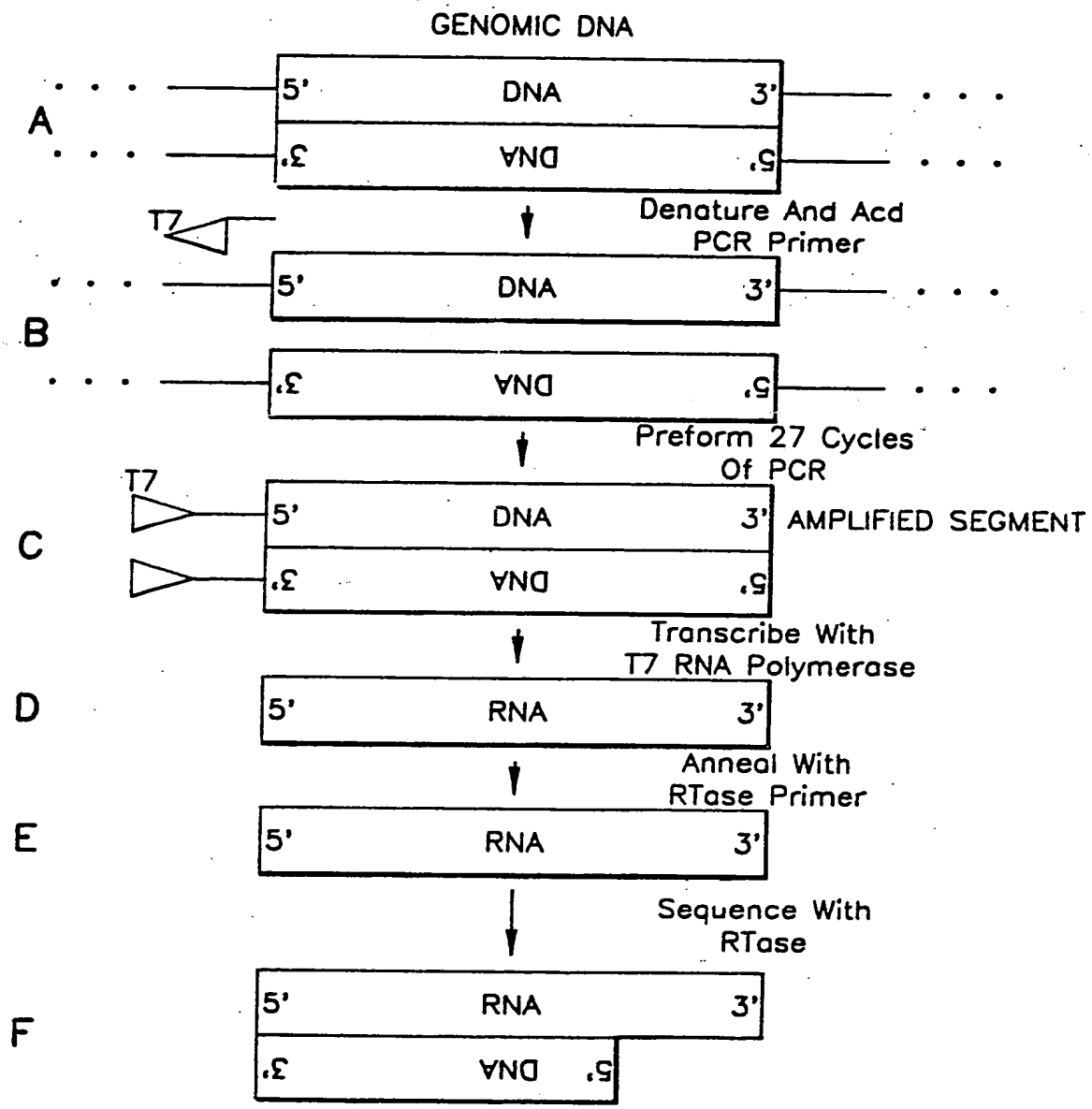


FIG. 2

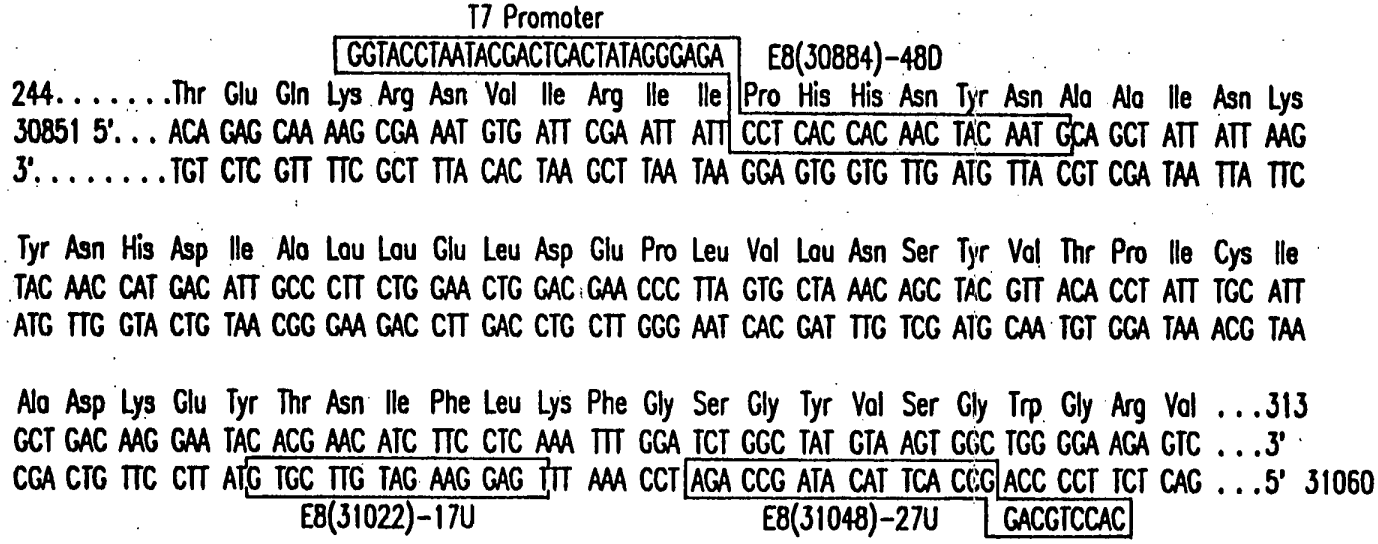


FIG. 3B

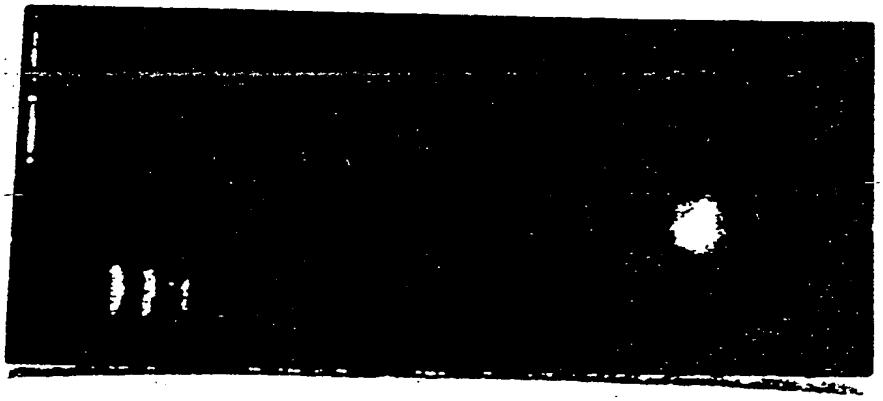


FIG. 3A

FIG. 3C

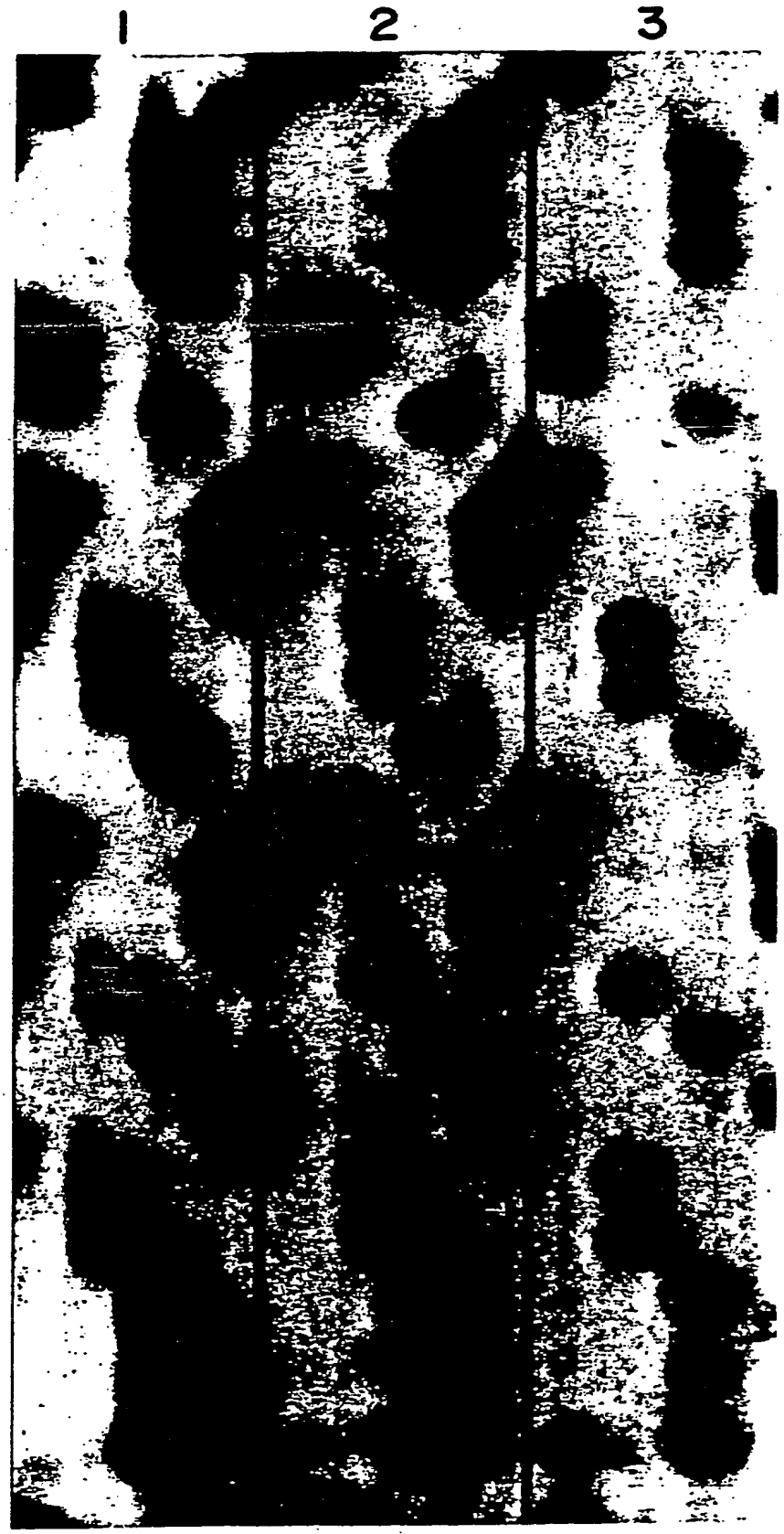
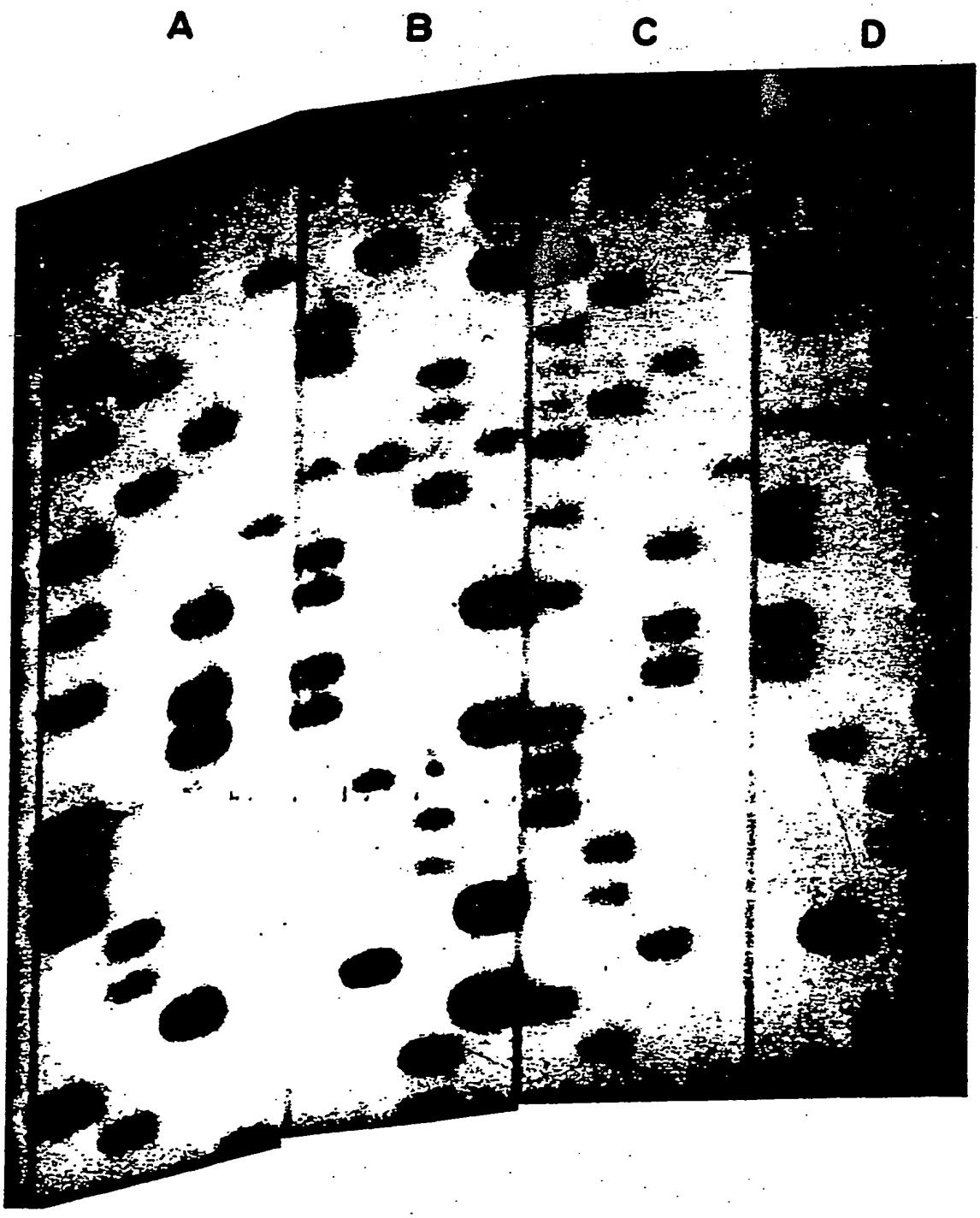


FIG. 4



INTERNATIONAL SEARCH REPORT

International Application No PCT/US89/00189

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): C12P 19/34; C12N 9/12; C 12Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/6, 91, 172.3, 193,194;536/27;935/16, 17, 18

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CA File: 1967-1989

Biosis File: 1967-1989

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	US, A, 4,683,202 (MULLIS) 28 July 1987. See the entire document.	1-7,21 8-20
Y	D.A. MELTON ET AL, "Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter", Nucleic Acids Research, Volume 12, Number 18, pages 7035-7056, published 1984 by IRL Press Limited (Oxford, England). See especially pages 7037-7039.	1-21
X Y	R.K. SAKAI ET AL, "Analysis of enzymatically amplified beta-globin and HLA-D Qalpha DNA with allele-specif oligonucleotide probes", Nature, Volume 324, pages 163-166, published 13 November 1986. by MacMillan Journals Ltd. (London, U.K.) See the entire document.	21 1-20

* Special categories of cited documents: [10]

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATI N

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 April 1989	15 MAY 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	James Martinell

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS C NSIDERED T BE RELEVANT (CONTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	S. YOSHITAKE ET AL, "Nucleotide sequence of the gene for human factor IX (anti-hemophilic factor B)", Biochemistry, Volume 24, pages 3736-3750, published 1985 by the American Chemical Society (Columbus, Ohio, USA) See the entire document.	8-20
X,P	E.S. STOFLET ET AL, "Genomic amplification with transcript sequencing," Science, Volume 239, pages 491-494, published 29 January 1988 by the American Association for the Advancement of Science (Washington, D.C., USA). See the entire document.	1-21